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(71) Applicant *(for all designated States except US)*: **CELL-TECH CHIROSCIENCE LIMITED** [GB/GB]; 216 Bath Road, Slough, Berkshire SL1 4EN (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **FINNEY, Helene, Margaret** [GB/GB]; 64 Clare Road, Maidenhead, Berkshire SL6 4DQ (GB). **LAWSON, Alastair, David, Griffiths** [GB/GB]; Holden Farm, Cheriton, Alresford, Hampshire SO2 0NX (GB).

(74) Agents: **MERCER, Christopher, Paul** et al.; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).

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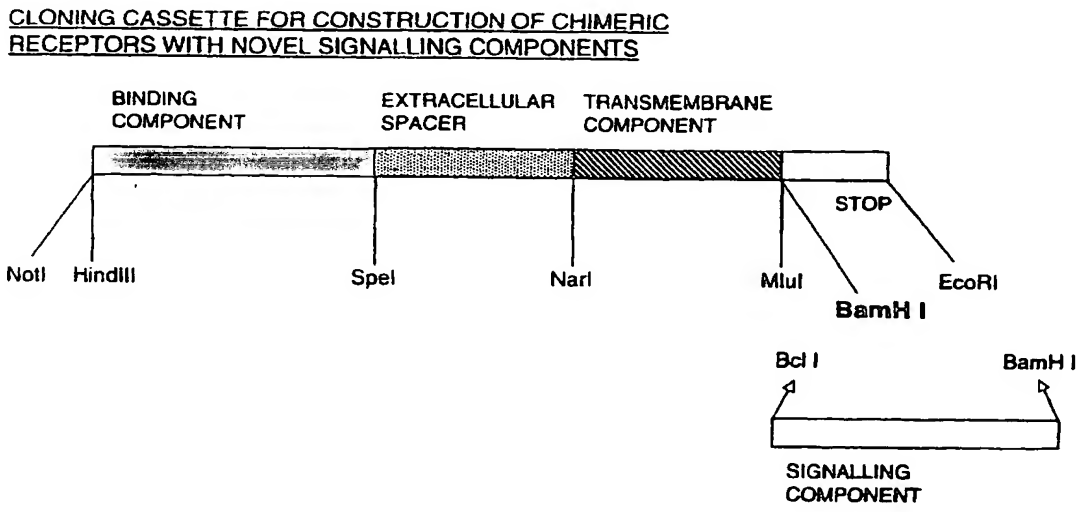

(57) Abstract: The invention relates to novel primary signalling motifs, which contain the consensus amino acid sequence of Y-X_2-L/I-X_n-Y-X_2-L/I, where n is 9 or greater. These novel motifs are extremely efficient at mediating immune cell signal transduction, particularly when incorporated in an intracellular signalling domain of a chimeric receptor. Nucleic acids that encode, and polypeptides that contain, such non-natural stimulatory primary signalling motifs are described, which are suitable for use in medicine.

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POLYPEPTIDES WITH EXPANDED PRIMARY SIGNALLING MOTIFS

The present invention relates to novel cytoplasmic signalling molecules, the nucleic acids that encode them, and the use of such polypeptides and nucleic acids in medicine and research.

Throughout this application various publications are referenced by author and year of publication. Full citations for these publications are provided following the detailed description of the invention and examples.

Research in the area of immune cell signalling has yielded a considerable amount of information about the signal transduction events that occur downstream of antigen receptor engagement. A substantial number of studies have concentrated on the receptors themselves, and the enzymes stimulated in response to antigen binding (reviewed by Weiss & Littman, 1994; DeFranco, 1997).

Individual components of the T cell receptor (TCR) complex have been well characterised and in a number of cases the functionality of receptor sub-units or domains has been determined though the construction of chimeric receptor proteins (Kuwana *et al.*, 1987; Romeo *et al.*, 1992). Cytoplasmic signalling domains in particular, and their role in TCR activation, have been identified using this approach. However, more recently such chimeric receptors have been used as regulators of the cell activation process (see for example published International Patent Specifications WO 97/23613 and WO 95/02686). The ability to control the biological effects of cellular activation, for example, increased cellular proliferation, increased expression of cytokines, stimulation of cytolytic activity, differentiation of other effector functions, antibody secretion, phagocytosis, tumour infiltration and/or increased cellular adhesion, with chimeric receptors has considerable therapeutic potential.

Whilst currently available chimeric receptors are capable of effectively activating cells, there is room for improvement in the efficacy with which the

cytoplasmic signalling domain of such a chimeric receptor transduces the signal from the extracellular ligand binding domain to downstream members of the secondary messenger pathway, such as members of the src and syk-tyrosine kinase family.

It would an advantage if transduction through such a cytoplasmic signalling domain could be improved or manipulated in order to achieve a required level of effector cell activation. It would also be of great benefit if cell activation could be effected with greater efficacy than is possible at present, thus regulating the induction or inhibition of any resultant biological processes.

The current invention addresses these difficulties by providing nucleic acids encoding novel cytoplasmic signalling sequences, which, when expressed in effector cells, are capable of efficiently regulating the level of cellular activation.

The term "cytoplasmic signalling sequence" as used herein, refers to cytoplasmic sequences of the TCR and co-receptors that act in concert to initiate signal transduction following antigen receptor engagement. The term also encompasses any derivative or variant of these sequences, and any synthetic sequence, that has the same functional capability. Signals generated through the TCR alone are insufficient for full activation of the T cell: a secondary or co-stimulatory signal is also required. Thus, cytoplasmic signalling components can be sub-divided into two classes: those that initiate antigen-dependent primary activation through the TCR (primary signalling sequences) and those that act in an antigen-independent manner to provide a secondary or co-stimulatory signal (secondary signalling sequences).

The first aspect of this invention provides a nucleic acid encoding a cytoplasmic signalling sequence, which comprises a primary signalling motif that has the consensus amino acid sequence of: $Y\text{-}X_2\text{-}L/I\text{-}X_n\text{-}Y\text{-}X_2\text{-}L/I$, wherein amino acid residues are represented by the standard single letter code, X represents any amino acid, a subscripted number indicates the number of residues present at that position within the motif and the value of n

is 9 or greater. It is intended that the terms X_2 or X_n can represent 2 or n amino acids (respectively) which may either be the same or different. It is also preferred that the value of n lies between 9 and 12, and the value of 9 is especially preferred.

The term "primary signalling motif" is defined as a sequence that tranduces either a stimulatory or an inhibitory signal, which regulates primary activation of the TCR complex. It will be appreciated that a primary signalling sequence may contain a single motif, or more than one such primary signalling motif. Naturally occurring, stimulatory, primary signalling motifs conform to the consensus sequence Y-X_2-L/I-X_n-Y-X_2-L/I where the value of n lies between 6 and 8 and is most typically found to be 7. These motifs are also known as immunoreceptor tyrosine-based activation motifs or ITAMs (Isakov, 1998).. By contrast, immunoreceptor tyrosine-based inhibitory motifs (ITIMs) comprising the consensus amino acid sequence of I/V-X-Y-X_2-L (Burshtyn *et al.*, 1999) are examples of inhibitory primary signalling motifs. It should be noted that, other than in Figure 2 and in some instances in the examples (where the standard three-letter code is used to describe amino acid sequence), the standard single letter code is used throughout this application to describe both amino acid and nucleotide sequences.

The present invention provides novel primary signalling motifs that are capable of modulating the level of primary signal transduction to different degrees and thus provide a range of cellular activation levels. It is known that primary activation involves the binding of src and the syk-family of protein tyrosine kinase to ITAMs and the subsequent phosphorylation of tyrosine residues within the motifs. It is likely therefore, that these novel primary signalling motifs of the invention mediate primary signal transduction through having an altered specificity or affinity for these kinases. This can be analysed quite easily through assay by Western blot using an anti-phosphotyrosine antibody (Sanchez-Garcia, *et al.*, 1997). Such an assay may be employed as the basis of a functional screen for the activity of novel primary signalling motifs of the invention.

The consensus sequence of the novel primary signalling motifs differs from that of naturally occurring ITAMs in that distance between the two tyrosine residues is increased by at least one amino acid (i.e. the new motifs conform to a new consensus of $Y\text{-}X_2\text{-}L/I\text{-}X_n\text{-}Y\text{-}X_2\text{-}L/I$, where the value of n is at least 9). These "expanded" motifs are able to signal at least as well as, if not better than, those with the natural consensus sequence. This is surprising since the corresponding single amino acid decrease in the inter-tyrosine distance (i.e. from n=6 to n=5) results in a considerable reduction in signalling ability of the primary signalling motif. Primary signalling motifs for use in this invention may be of variable sequence, provided that they conform to the consensus sequence of $Y\text{-}X_2\text{-}L/I\text{-}X_n\text{-}Y\text{-}X_2\text{-}L/I$ where the value of n is 9 or greater. However, preferred motifs are those where the value of n is 9 and particularly preferred motifs are SBX^a (which has the amino acid sequence: RKNPQEGLYNELQKDKMAEDTYDALHMQA), $SBQ9^a$ (which has the amino acid sequence: GQNQLYNELQQQQQQQQQYDVLRRGRDPEM) and $SB16^a$ (which has the amino acid sequence: GQDGLYQELNTRSRDEAAYSVLEGR KAR). Further examples are given in Figure 4 as SBX, SBQ9 and SB16, where GS linkers have additionally been incorporated at each end of the sequence to facilitate cloning.

It has also been found that when novel cytoplasmic signalling motifs of the invention are employed as the intracellular domain of a chimeric receptor, the magnitude of a signal transduced through an immune cell receptor may be tailored further by incorporating additional cytoplasmic signalling motifs and/or sequences within the intracellular domain. Thus, in a second aspect of the invention, a nucleic acid is provided that encodes a cytoplasmic signalling molecule comprising a primary signalling motif according to the first aspect of the invention and at least one other primary signalling motif.

Primary signalling motifs for use in this aspect of the invention may contain the consensus amino acid sequence $Y\text{-}X_2\text{-}L/I\text{-}X_n\text{-}Y\text{-}X_2\text{-}L/I$ (single letter code). Where the value of n is 9, SBX^a, $SBQ9^a$ or $SB16^a$, may be employed as the additional motif(s). Where the value of n lies between 6 and 8, it is preferred

that the at least one other primary signalling motif will comprise at least part of an immunoreceptor tyrosine based activation motif (ITAM), for example one derived from the TCRζ chain, FcRγ, FcRβ, CD3γ, CD3δ, CD3ε, CD5, CD22, CD79a, CD79b or CD66d. Examples of primary signalling motifs derived from these molecules are shown in Table 1 below. Preferably the at the least one other primary signalling motif for use in this aspect of the invention will be derived from all or part of TCRζ1, TCRζ2, TCRζ3, FcRγ, FcRβ, CD3γ, CD3δ, CD5, CD22, CD79a, CD79b or CD66d.

Table 1. Source and amino acid sequences of primary signalling motifs of particular use in the invention. The position of the consensus amino acid sequence is emphasised in bold. Figure 4 includes further examples of primary signalling motifs for use in the invention (SB1, SB2, SB3, SB4, SB4*, SB5, SB6, SB7, SB8, SB9, SB10, SB11, SB12, SB13, SB14, SB15, SB16, SBX and SBQ9), which correspond to the primary signalling motifs shown below with GS linkers incorporated at each end of the motif to facilitate cloning.

Source	Primary Signalling Motif	Amino Acid Sequence
TCRζ1	SB1[a]	GQNQL**YNELNLGRREEYDVL**DKRRGRDPEM
TCRζ2	SB2[a]	RKNPQEGL**YNELQKDKMAEAYSEI**GMKGER
TCRζ3	SB3[a]	RGKGHDGL**YQGLSTATKDTYDAL**HMQA
FcRγ	SB4[a]	YEKSDGV**YTGLSTRNQETYETL**KHEKP
FcRβ	SB5[a]	GNKBPEDRV**YEELNIYSATYSEL**EDPGEMSP
CD3γ	SB6[a]	KQTLLPNDQL**YQPLKDREDDQYSHL**QGNQLR
CD3δ,	SB7[a]	ALLRNDQV**YQPLRDRDDAQYSHL**GGNWARNK
CD3ε	SB8[a]	QNKERPPPVPNPD**YEPIRKGQRDLYSGL**NQRRI
CD5	SB9[a]	HVDNE**YSQPPRNSRLSAYPAL**EGVLHRS
CD22	SB10[a]	PPRTCDDTVT**YSALHKRQVGDYENV**IPDFPEDE
CD79a	SB11[a]	EYEDENL**YEGLNLDDCSMYEDI**SRGLQGTYQDV
CD79b	SB12[a]	KAGMEEDHT**YEGLDIDQTATYEDI**VTLRTGEV
CD66d	SB13[a]	PLPNPRTAASI**YEELLKHDTNIYCRM**DHKAEVA
FcRγ	SB4*[a]	YEKSDGV**YTGLSTRNQETYDTL**KHEKP
Non-natural	SB14[a]	GQDGL**YQELNTRSRDEYSVL**EGRKAR
Non-natural	SB15[a]	GQDGL**YQELNTRSRDEAYSVL**EGRKAR
Non-natural	SB16[a]	GQDGL**YQELNTRSRDEAAYSVL**EGRKAR
Non-natural	SBX[a]	RKNPQEGL**YNELQKDKMAEDTYDAL**HMQA
Non-natural	SBQ9[a]	GQNQL**YNELQQQQQQQQQYDVL**RRGRDPEM

Alternatively, at least one of the additional primary signalling motifs will be non-natural but still conform to the consensus amino acid sequence of sequence Y-X_2-L/I-X_{6-8}-Y-X_2-L/I. By "non-natural ITAM" is meant a synthetic primary signalling motif, the sequence of which has not been found in nature. Preferred examples of such non-natural primary signalling motifs will be SB4*[a], SB14[a] or SB15[a] as described herein in Table 1 above, or non-natural variants thereof.

Preferred combinations of motifs according to this aspect of the invention include multiples of SBQ9[a], SBQX[a], or SB16[a]; SBQ9[a], SBX[a] or SB16[a] in combination with each other, or in combination with a primary signalling motif(s) derived from the TCRζ chain (i.e. SB1[a], SB2[a] or SB3[a]), or in combination with SB4*[a]. The combinations of SB16[a] with SB2[a], SB4*[a] and SB4[a], and SBX[a] with SB3[a], are particularly preferred.

In a third aspect of the invention, a nucleic acid is provided that encodes a cytoplasmic signalling molecule comprising a primary signalling motif according to the first aspect of the invention and at least one secondary signalling sequence.

The term "secondary signalling sequence" means a sequence that imparts secondary or co-stimulatory signalling capacity to a molecule in T cells. Molecules containing such sequences include CD2, CD4, CD8, CD28, CD134 and CD154 (see Finney *et al.*, 1998). Preferred secondary signalling sequences for use in the invention are those derived from CD28, CD134 and CD154, for example, SB28[a], which has the amino acid sequence RLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFA, SB29[a], which has the amino acid sequence MIETYNQTSPRSAATGLPISMK and SB34[a], which has the amino acid sequence RRDQRLPPDAHKPPGGGSFRTPIQEEQADAHS TLAKI. Further examples of secondary signalling sequences are shown in Figure 4 as SB28, SB29 and SB34, where GS linkers have been incorporated at each end of the sequence to facilitate cloning.

Preferred combinations of primary signalling motifs and secondary signalling sequence(s) according to this aspect of the invention include $SBQ9^a$, SBX^a or $SB16^a$ in combination with $SB28^a$. The combination of $SBQ9^a$ with $SB28^a$ is particularly preferred.

Primary signalling motifs may be combined with each other or with secondary signalling sequences in a random or specified order. Optionally, there will be a short oligo- or polypeptide linkage between component motifs/sequences that will preferably be between 2 and 10 amino acids in length. A glycine-serine doublet provides a particularly suitable linker.

Novel cytoplasmic signalling sequences and molecules of the invention can be used either by themselves or as a component part of a larger protein, such as a chimeric receptor. As individual protein molecules they can be introduced into, or expressed in, effector cells in order to act as substitute cytoplasmic signalling sequences for immune cell receptors already expressed within that cell. In this way they can increase the efficiency of signalling through the receptor. Alternatively, they may be introduced into a cell in order to compete with the existing src and syk-protein kinase binding sites and thus, regulate the degree of cell activation by acting as protein kinase inhibitors. Any one of a number of *in vitro* assays may be used to estimate the efficacy of these novel cytoplasmic signalling molecules as protein tyrosine kinase inhibitors, see for example published International Patent specification WO98/11095.

However, it is envisaged that the cytoplasmic signalling sequences and molecules of this invention are used preferentially to mediate signalling when employed as an intracellular domain of a chimeric receptor protein. Such chimeric receptors additionally comprise an extracellular ligand-binding domain and a transmembrane domain.

The incorporation of an extracellular ligand-binding domain confers on the chimeric receptor the ability to exhibit specificity for a specific ligand or class of ligands. This specificity can be used to define precise ligands or classes

of ligands that are capable of activating the receptor. In this way the receptor may be designed to activate the cell in which it is expressed upon binding a chosen class of, or individual, ligand.

Contact between the ligand and its corresponding binding domain in a chimeric receptor, results in signal transduction through the cytoplasmic signalling domain. The choice of motif, or combination of motifs, or motif(s) and sequence(s) within the cytoplasmic signalling domain, dictates the magnitude of the signal transduced, and consequently controls the level to which the cell is activated.

A further embodiment of the invention thus provides a nucleic acid encoding a chimeric receptor protein that comprises an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic signalling domain, wherein the cytoplasmic signalling domain is encoded by a nucleic acid according to any one of the previously described aspects of the invention.

The term "extracellular ligand-binding domain" as used herein, is defined as any oligo- or polypeptide that is capable of binding a ligand. Accordingly antibody binding domains, antibody hypervariable loops or CDRs, receptor binding domains and other ligand binding domains, examples of which will be readily apparent to the skilled artisan, are described by this term. Preferably the domain will be capable of interacting with a cell surface molecule. Examples of proteins associated with binding to cell surface molecules that are of particular use in this invention include, antibody variable domains (V_H or V_L), T-cell receptor variable region domains (TCRα, TCRβ, TCRγ, TCRδ), or the chains of CD8α, CD8b, CD11A CD11B, CD11C, CD18, CD29, CD49A, CD49B, CD49D, CD49E, CD49F, CD61, CD41, or CD51. Whilst it may be of benefit to use the entire domain or chain in some instances, fragments may be used where appropriate.

Particularly useful binding components are derived from antibody binding domains and include Fab' fragments and especially single chain Fv fragments.

The choice of domain will depend upon the type and number of ligands that define the surface of a target cell. For example, the extracellular ligand binding domain may be chosen to recognise a ligand that acts as a cell surface marker on target cells associated with a particular disease state. Thus examples of cell surface markers that may act as ligands include those associated with viral, bacterial and parasitic infections, autoimmune disease and cancer cells. In the latter case, specific examples of cell surface markers are the bombesin receptor expressed on lung tumour cells, carcinoembryonic antigen (CEA), polymorphic epithelial mucin (PEM), CD33, the folate receptor, epithelial cell adhesion molecule (EPCAM) and *erb*-B2. Other ligands of choice are cell surface adhesion molecules, inflammatory cells present in autoimmune disease, and T-cell receptors or antigens that give rise to autoimmunity. The potential ligands listed above are included by way of example; the list is not intended to be exclusive and further examples will be readily apparent to those of skill in the art.

Chimeric receptors may be designed to be bi- or multi-specific i.e. they may comprise more than one ligand binding domain and therefore, be capable of exhibiting specificity for more than one ligand. Such receptors may recruit cellular immune effector cells (e.g. T cells, B cells, natural killer (NK) cells, macrophages, neutrophils, eosinophils, basophils, or mast cells), or components of the complement cascade.

A further component of a chimeric receptor is the transmembrane domain. This may be derived either from a natural or from a synthetic source. Where the source is natural, the domain may be derived from any membrane-bound or transmembrane protein. Transmembrane regions of particular use in this invention may be derived from (i.e. comprise at least the transmembrane region(s) of) the α, β or ζ chain of the T-cell receptor, CD28, CD3ε, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 or CD154. It is preferable that the transmembrane domain is derived from all or part of the α, β or ζ chain of the T-cell receptor, CD28,

CD3ε, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, or CD154. The use of a transmembrane domain derived from CD28 is particularly preferred. Alternatively the transmembrane domain may be synthetic, in which case it will comprise predominantly hydrophobic residues such as leucine and valine (see for example Published International Patent Specification WO00/63374). Preferably a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain.

Between the extracellular ligand-binding domain and the transmembrane domain, or between the cytoplasmic signalling domain and the transmembrane domain, there may be incorporated a spacer domain. As used herein, the term "spacer domain" generally means any oligo- or polypeptide that functions to link the transmembrane domain to, either the extracellular ligand-binding domain or, the cytoplasmic signalling domain in the polypeptide chain. A spacer domain may comprise up to 300 amino acids, preferably 10 to 100 amino acids and most preferably 25 to 50 amino acids.

Spacer domains may be derived from all or part of naturally occurring molecules, such as from all or part of the extracellular region of CD8, CD4, or CD28; all or part of an antibody constant region; all or part of natural spacer components between functional parts of cytoplasmic signalling components, for example spacers between ITAMs. Alternatively, the spacer may be an entirely synthetic spacer sequence.

Spacer domains may be designed in such a way that they, either minimise the constitutive association of chimeric receptors, thus reducing the incidence of constitutive activation in the cell or, promote such associations and enhance the level of constitutive activation in the cell. Either possibility may be achieved artificially by deleting, inserting, altering or otherwise modifying amino acids and naturally occurring sequences in the transmembrane and/or spacer domains, which have side chain residues that are capable of covalently or non-covalently interacting with the side chains of amino acids in

other polypeptide chains. Particular examples of amino acids that can normally be predicted to promote association include cysteine residues, charged amino acids or amino acids such as serine or threonine within potential glycosylation sites.

Optionally, cytoplasmic signalling molecules of the invention may be linked to other intracellular components, or to the transmembrane domain through a short oligo- or polypeptide linker, preferably between 2 and 10 amino acids. A glycine-serine doublet provides a particularly suitable linker.

Chimeric receptors may be designed in such a way that the spacer and transmembrane components have free thiol groups, thereby providing the receptor with multimerisation, and particularly dimerisation, capacity. Such multimeric receptors are preferred, especially dimers. Receptors with transmembrane and spacer domains derived from CD28 components, the zeta chain of the natural T cell receptor, and/or antibody hinge sequences are especially preferred.

It is intended that the component motifs and sequences of the novel cytoplasmic signalling molecules of the invention can be combined in any way so as to achieve the desired level of activation (or possible inhibition) of a number of secondary messenger cascades. When a cytoplasmic signalling motif/molecule of the invention is employed as the intracellular domain of a chimeric receptor, this activation/inhibition may be initiated though a single binding event at the extracellular ligand-binding domain. It will be clear to the skilled technician that combinations of cytoplasmic signalling motifs, or motifs and sequences, can be on separate polypeptide chains or may be in series on a single polypeptide chain. This concept of either a single polypeptide chain, or multiple polypeptide chains, providing the comprising elements, is equally applicable to the chimeric receptors of the present invention.

Thus the current invention also provides novel cytoplasmic signalling proteins and chimeric receptor proteins encoded by a nucleic acid as described in any of the previously described aspects of the invention.

Nucleic acid coding sequences for the novel primary signalling motifs, for use in this invention, are readily derived from the specified amino acid sequences. Other nucleic acid sequences are widely reported in the scientific literature and are also available in public databases. DNA may be commercially available, may be part of cDNA libraries, or may be generated using standard molecular biology and/or chemistry procedures as will be clear to those of skill in the art. Particularly suitable techniques include the polymerase chain reaction (PCR), oligonucleotide-directed mutagenesis, oligonucleotide-directed synthesis techniques, enzymatic cleavage or enzymatic filling-in of gapped oligonucleotide. Such techniques are described by Sambrook & Fritsch, 1989, and in the Examples contained hereinafter.

The nucleic acids of the invention may be used with a carrier. The carrier may be a vector or other carrier suitable for the introduction of the nucleic acids *ex-vivo* or *in-vivo* into target cell and/or target host cells. Examples of suitable vectors include viral vectors such as retroviruses, adenoviruses, adeno-associated viruses (AAVs), Epstein-Barr virus (EBV) and Herpes simplex virus (HSV). Non-viral vector may also be used, such as liposomal vectors and vectors based on condensing agents such as the cationic lipids described in International patent application numbers WO96/10038, WO97/18185, WO97/25329, WO97/30170 and WO97/31934. Where appropriate, the vector may additionally include promoter and regulatory sequences and/or replication functions from viruses, such as retrovirus long terminal repeats (LTRs), AAV repeats, SV40 and human cytomegalovirus (hCMV) promoters and/or enhancers, splicing and polyadenylation signals and EBV and BK virus replication functions. Tissue-specific regulatory sequences such as the TCR-α promoter, E-selectin promoter and the CD2 promoter and locus control region may also be used. The carrier may be an antibody.

The invention also includes cloning and expression vectors containing a nucleic acid according to any of the above-described aspects of the

invention. Such expression vectors will incorporate the appropriate transcriptional and translation control sequences, for example, enhancer elements, promoter-operator regions, termination stop sequence, mRNA stability sequences, start and stop codons or ribosome binding sites, linked where appropriate in-frame with the nucleic acid molecules of the invention.

Additionally in the absence of a naturally effective signal peptide in the protein sequence, it may be convenient to cause recombinant cytoplasmic signalling proteins to be secreted from certain hosts. Accordingly, further components of such vectors may include nucleic acid sequences encoding secretion signalling and processing sequences.

Vectors according to the invention include plasmids and viruses (including both bacteriophage and eukaryotic viruses). Many expression systems suitable for the expression of heterologous proteins are well known and documented in the art. For example, the use of prokaryotic cells such as *Escherichia coli* to express heterologous polypeptides and polypeptide fragments is well established (see for example, Sambrook & Fritsch, 1989, Glover, 1995a). Similarly, eukaryotic expression systems have been well developed and are commonly used for heterologous protein expression (see for example, Glover, 1995b and O'Reilly *et al.*, 1993). In eukaryotic cells, apart from yeasts, the vectors of choice are virus-based. Particularly suitable viral vectors include baculovirus-, adenovirus-, and vaccinia virus-based vectors.

Vectors containing the relevant regulatory sequences (including promoter, termination, polyadenylation, and enhancer sequences, marker genes) can either be chosen from those documented in the literature, or readily constructed for the expression of the proteins of this invention using standard molecular biology techniques. Such techniques, and protocols for the manipulation of nucleic acids, for example in the preparation of nucleic acid constructs, mutagenesis, sequencing, DNA transformation and gene expression, as well as the analysis of proteins, are described in detail in Ausubel *et al.*, 1992 or Rees *et al.*, 1993.

Suitable host cells for the *in vitro* expression of high levels of cytoplasmic signalling molecules or chimeric receptors include prokaryotic cells e.g. *E. coli*, eukaryotic yeasts e.g *Saccharomyces cerevisiae*, *Pichia* species, *Schizosaccharomyces pombe*, mammalian cell lines and insect cells. Alternatively recombinant proteins or chimeric receptors may be expressed *in vivo*, for example in insect larvae, or plant cells or more preferably in mammalian tissues.

Nucleic acid may be introduced into a host cell by any suitable technique. In eukaryotic cells these techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, particle bombardment, liposome-mediated transfection or transduction using retrovirus, adenovirus or other viruses, such as vaccinia or, for insect cells, baculovirus. In bacterial cells, suitable techniques may include calcium chloride transformation, electroporation or transfection using bacteriophage. The nucleic acid may remain in an episomal form within the cell, or it may integrate into the genome of the cell. If the latter is desired, sequences that promote recombination with the genome will be included in the nucleic acid. Following introduction of the nucleic acid into host cells, the cells may be cultured under conditions to enhance or induce expression of the recombinant cytoplasmic signalling molecule or chimeric receptor protein as appropriate.

Thus, further aspects of the invention provide host cells containing a nucleic acid encoding a cytoplasmic signalling protein and/or chimeric receptor protein of the invention, and host cells expressing such proteins.

In further embodiments the nucleic acids of the invention be employed in either *ex-vivo* or *in-vivo* therapies.

For *ex-vivo* use, the nucleic acid may be introduced into effector cells, removed from the target host, using methods well known in the art e.g. transfection, transduction (including viral transduction), biolistics, protoplast fusion, calcium phosphate mediated DNA transformation, electroporation,

cationic lipofection, or targeted liposomes. The effector cells are then reintroduced into the host using standard techniques. Examples of suitable effector cells for the expression of the chimeric receptors of the present invention include cells associated with the immune system such as lymphocytes e.g. cytotoxic T-lymphocytes, tumour infiltrating lymphocytes, neutrophils, basophils, or T-helper cells, dendritic cells, B-cells, haematopoietic stem cells, macrophages, monocytes or NK cells. The use of cytotoxic T-lymphocytes is especially preferred.

The nucleic acid according to this aspect of the invention is particularly suitable for *in vivo* administration. In order to achieve this, the DNA may be in the form of a targeted carrier system in which a carrier as described above is capable of directing DNA to a desired effector cell. Examples of suitable targeted delivery systems include targeted naked DNA, targeted liposomes encapsulating and/ or complexed with the DNA, targeted retroviral systems and targeted condensed DNA such as protamine and polylysine-condensed DNA.

Targeting systems are well known in the art and include, for example, using antibodies or fragments thereof against cell surface antigens expressed on target cells in vivo such as CD8, CD16, CD4, CD3, selecting (e.g. E-selectin), CD5, CD7, CD24, and activation antigens (e.g. CD69 and IL-2R. Alternatively other receptor-ligand interactions can be used for targeting e.g. CD4 to target $HIV_{gp}160$-expressing target cells.

In general, the use of antibody-targeted DNA is preferred, particularly antibody-targeted naked DNA, antibody-targeted condensed DNA and especially antibody-targeted liposomes. Types of liposomes that may be used include for example pH-sensitive liposomes, where linkers that are cleaved at low pH may be used to link the antibody to the liposome. The nucleic acids of the present invention may also be targeted directly to the cytoplasm by using cationic liposomes, which fuse with the cell membrane. Liposomes for use in the invention may also have hydrophilic molecules, e.g. polyethylene glycol polymers, attached to their surface to increase their

circulating half-life. There are many example in the art of suitable groups for attaching DNA to liposomes or other carriers; see for example International patent application numbers WO88/04924, WO90/09782, WO91/05545, WO91/05546, WO93/19738, WO94/20073 and WO94/22429. The antibody or other targetting molecule may be linked to the DNA, condensed DNA or liposome using conventional linking groups and reactive functional groups in the antibody, e.g. thiols or amines, and in the DNA or DNA-containing material.

Non-targeted carrier systems may also be used. In these systems targeted expression of the protein is advantageous. This may be achieved, for example, by using T cell specific promoter systems such as the zeta promoter, CD2 promoter and locus control region, CD4, CD8 TCRα and TCRβ promoters, cytokine promoters, such as the IL2 promoter, and the perforin promoter.

It is intended that cytoplasmic signalling proteins and/or chimeric receptor proteins of the present invention, or the nucleic acids encoding them, be applied in methods of therapy of mammalian, particularly human, patients. Signalling molecules and chimeric receptor proteins generated by the present invention may be particularly useful in the treatment of a number of diseases or disorders. Such diseases or disorders may include those described under the general headings of infectious diseases, e.g. HIV infection; inflammatory disease/autoimmunity e.g. asthma, eczema; congenital e.g. cystic fibrosis, sickle cell anaemia; dermatologic, e.g. psoriasis; neurologic, e.g. multiple sclerosis; transplants e.g. organ transplant rejection, graft-versus-host disease; metabolic/idiopathic disease, e.g. diabetes; cancer.

For example, expression of a chimeric receptor on the surface of a T cell may initiate the activation of that cell upon binding of the ligand-binding domain to a ligand on a target cell. The ensuing release of inflammatory mediators stimulated by the activation of the signalling function of the receptor ensures destruction of the target cell.

When a chimeric receptor according to the present invention is expressed in an effector cell of the immune system, binding to target will activate the effector cell; downstream effects of this activation may also result in the destruction of the target cell. If the extracellular ligand-binding domain of the chimeric receptor exhibits specificity for a surface marker on an immune cell, effector cells may be recruited to the site of disease. Accordingly, expression of a chimeric receptor in a diseased cell will ensure its destruction.

The expression of multispecific chimeric receptor proteins, or more than one chimeric receptor (with different ligand specificities), within a single host cell, may confer dual functionality on the receptor. For example, binding of the chimeric receptor to its target may not only activate the effector cell itself, but may additionally attract other immune effectors to the site of disease. The target cell may thus be destroyed by the activation of the immune system.

A further aspect of the invention provides a composition comprising a cytoplasmic signalling protein and/or chimeric receptor protein of the invention, or nucleic acid(s) encoding such a protein, in conjunction with a pharmaceutically acceptable excipient.

Suitable excipients will be well known to those of skill in the art and may, for example, comprise a phosphate-buffered saline (e.g. 0.01M phosphate salts, 0.138M NaCl, 0.0027M KCl, pH7.4), a liquid such as water, saline, glycerol or ethanol, optionally also containing mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulphates and the like; and the salts of organic acids such as acetates propionates, malonates, benzoates and the like. Auxiliary substances such as wetting or emulsifying agents, and pH buffering substances, may also be present. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991). Preferably, the compositions will be in a form suitable for parenteral administration e.g. by injection or infusion, for example by bolus injection or continuous infusion or particle-mediated injection. Where the composition is for injection or infusion, it may take the form of a suspension, solution or emulsion in an oily or aqueous vehicle and it may

contain formulatory agents such as suspending, preservative, stabilising and/or dispersing agents. Alternatively, the composition may be in dry form, for reconstitution before use with an appropriate sterile liquid. For particle-mediated administration, DNA may be coated on particles such as microscopic gold particles.

A carrier may also be used that does not itself induce the production of antibodies harmful to the individual receiving the composition and which may be administered without undue toxicity. Suitable carriers are typically large, slowly metabolised macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers and inactive virus particles. Pharmaceutical compositions may also contain preservatives in order to prolong shelf life in storage.

If the composition is suitable for oral administration, the formulation may contain, in addition to the active ingredient additives such as starch (e.g. potato, maize or wheat starch, cellulose), starch derivatives such as microcrystalline cellulose, silica, various sugars such as lactose, magnesium carbonate and/or calcium phosphate. It is desirable that a formulation suitable for oral administration be well tolerated by the patient's digestive system. To this end, it may be desirable to include mucus formers and resins. It may also be desirable to improve tolerance by formulating the compositions in a capsule that is insoluble in the gastric juices. In addition, it may be preferable to include the composition in a controlled release formulation.

According to yet a further aspect of the invention, the use of novel cytoplasmic signalling proteins or chimeric receptor proteins, or the nucleic acids encoding such proteins, or of a pharmaceutical composition, in the manufacture of a medicament for the treatment or prevention of disease in humans or in animals is also provided.

The various aspects and embodiments of the present invention will now be illustrated in more detail by way of example. It will be appreciated that

modification of detail may be made without departing from the scope of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 Schematic representation of the cloning cassette used in the construction of chimeric receptors with novel signalling components.

Figure 2 Nucleotide and amino acid sequence of h.CD28 extracellular spacer and the human CD28 transmembrane region used in the construction of the cloning cassette describe in Figure 1.

Figure 3 Oligonucleotide sequences used in the construction of chimeric receptors.

Figure 4 Amino acid sequences of primary and secondary signalling sequences employed in the construction of chimeric receptors.

Figure 5 Antigen specific stimulation of chimeric receptors with primary signalling motifs that vary in their inter-tyrosine distance.

Figure 6 Antigen specific stimulation of a chimeric receptor with an expanded primary signalling motif in conjunction with an addition primary signalling motif.

Figure 7 Antigen specific stimulation of chimeric receptors that comprise primary signalling motifs, which vary in their inter-tyrosine distance, in conjunction with a secondary signalling sequence.

EXAMPLES

Example 1. Construction of the cloning vector, pHMF393

To facilitate construction of chimeric receptors with different binding, extracellular spacer, transmembrane and signaling components, a cloning cassette system was devised in pBluescript SK+ (Stratagene). This is a modification of our cassette system described in published International Patent specification WO97/23613.

This new cassette system is shown in Figure 1. The binding component has 5' (relative to coding direction) Not I and Hind III restriction sites and a 3' (again relative to coding direction) Spe I restriction site. The extracellular spacer is

flanked by a Spe I site (therefore encoding Thr, Ser at the 5' end) and a Nar I site (therefore encoding Gly, Ala at the 3' end). The transmembrane component is flanked by a Nar I site at its 5' end (therefore encoding Gly, Ala) and by Mlu I (therefore encoding Thr, Arg) and BamH I sites (therefore encoding Gly, Ser) at the 3' end. The signalling component may be cloned in-frame into the BamH I site. Following this BamH I site there is a stop codon for transcription termination and there is also an EcoR I site situated downstream of this to facilitate the subsequent rescue of whole constructs.

To generate the cassette, a 200bp fragment was assembled by PCR, using the following oligos; S0146, A6081, A6082 and A6083 (Figure 3). The nucleotide and amino acid sequences of this fragment are shown in Figure 2. It starts with a SpeI site and consists of the extracellular spacer h.CD28, the human CD28 transmembrane region, a stop codon and finishes with an EcoR I restriction site. This PCR fragment was then digested with Spe I and EcoR I and substituted for the same fragment in our previously described cloning cassette system (Figure 2 of published International Patent application WO 97/23613) in order to clone it in-frame with the binding component.

Example 2. The construction of sequence blocks of primary and secondary signalling motifs

Each sequence block (SB) was generated by annealing two oligos such that they had single-stranded overhangs forming half a Bcl I site at the 5' end and half a BamH I site at the 3' end. Oligos were annealed at a concentration of 1pmole/µl in buffer (25mM NaCl, 12.5 mM Tris-HCl, 2.5mM $MgCl_2$, 0.25mM DTE, pH 7.5) by heating in a boiling water bath for 5 minutes and then allowing them to cool slowly to room temperature.

The predicted amino acid sequences of these examples of SBs are shown in Figure 4 and sequence of the oligonucleotides employed in their construction are given in Figure 3. All oligonulceotides were phosphorylated at their 5' end.

a) SB1: This sequence is based on the first ITAM of human TCRζ and was constructed by annealing oligos A8816 and A8817.

b) SB2: This sequence is based on the second ITAM of human TCRζ and was constructed by annealing oligos A8814 and A8815.

c) SB3: This sequence is based on the third ITAM of human TCRζ and was constructed by annealing oligos A8812 and A8813.

d) SB4: This sequence is based on the ITAM of the γ chain of human FcεR1 and was constructed by annealing oligos A8810 and A8811.

e) SB4*: This sequence was originally generated in error by mis-annealment of the above oligos but was subsequently made by annealing oligos A8810B and A8811B.

f) SB5: This sequence is based on the ITAM of the β chain of human FcεR1 and was constructed by annealing oligos A9000 and A9001.

g) SB6: This sequence is based on the ITAM of the γ chain of human CD3 and was constructed by annealing oligos A9002 and A9003.

h) SB7: This sequence is based on the ITAM of the δ chain of human CD3 and was constructed by annealing oligos A9004 and A9005.

i) SB8: This sequence is based on the ITAM of the ε chain of human CD3 and was constructed by annealing oligos A9006 and A9007.

j) SB9: This sequence is based on the ITAM of human CD5 and was constructed by annealing oligos A9008 and A9009.

k) SB10: This sequence is based on the ITAM of human CD22 and was constructed by annealing oligos A9010 and A9011.

l) SB11: This sequence is based on the ITAM of human CD79a and was constructed by annealing oligos A9012 and A9013.

m) SB12: This sequence is based on the ITAM of human CD79b and was constructed by annealing oligos A9014 and A9015.

n) SB13: This sequence is based on the ITAM of human CD66d and was constructed by annealing oligos A9016 and A9017.

o) SB14: This sequence is synthetic and was constructed by annealing oligos D5258 and D5259.

p) SB15: This sequence is synthetic and was constructed by annealing oligos F6392 and F6394.

q) SB16: This sequence is synthetic and was constructed by annealing oligos F6393 and F6395.

r) SB28: This sequence is based on the secondary signalling (costimulation) sequence of human CD28 and was constructed by annealing oligos A9018 and A9019.

s) SB29: This sequence is based on the secondary signalling (costimulation) sequence of human CD154 and was constructed by annealing oligos A9020 and A9021.

t) SB34: This sequence is based on the secondary signalling (costimulation) sequence of human CD134 and was constructed by annealing oligos F1340A and F1340B.

u) SBQ5: This sequence is synthetic and was constructed by annealing oligos D7609 and D7610.

v) SBQ7: This sequence is synthetic and was constructed by annealing oligos D7613 and D7614.

w) SBQ9: This sequence is synthetic and was constructed by annealing oligos D7617 and D7618.

x) SBX: This sequence resulted from a recombination event between SB2 and SB3 during cloning.

Example 3. The construction of receptors with different signalling components.

Primary signalling motifs and secondary signalling sequences (in the form of SBs, see Figure 4) on a Bcl I to BamH I fragment may be cloned into the BamH I site of the cassette described in Example 1, downstream of the transmembrane region. Cloning in the correct orientation allows the subsequent downstream insertion of other signalling motifs/sequences.

This cassette also facilitates exchange of binding components on a Not I or Hind III to Spe I fragment as well as the exchange of extracellular spacers on a Spe I to Nar I fragment and the exchange of the transmembrane region on a Nar I to Mlu I fragment. Thus, chimeric receptors with different binding, extracellular spacer, transmembrane and signalling components can be assembled readily.

Example 4. Analysis of chimeric receptors

a) Construction of expression plasmids. The chimeric receptor constructs were sub-cloned from pBluescript KS+ into the expression vector pEE6hCMV.ne (Cockett, *et al.*, 1991) on a Hind III to EcoR I restriction fragment. The empty expression vector (i.e the base vector lacking in chimeric receptor genes) is used as a negative control.

b) Transfection into Jurkat E6.1 cells. To generate stable cell lines, the expression plasmids were linearised and transfected into Jurkat E6.1 cells (ECACC) by electroporation using a BioRad Gene Pulser. Cells (~2.5 $X10^6$)

were mixed with DNA (10μg) and pulsed twice at 1kV, 3μF (0.4cm electrode gap cuvette) in 1ml PBS. The cells were left to recover overnight in non-selective media before being selected and cultured in media supplemented with the antibiotic G418 (Sigma) at 1.5mg/ml. After approximately four weeks cells were ready for analysis.

For transient expression in Jurkat cells, the expression plasmids were transfected using DuoFect (Quantum Biotechnologies Inc.) according to the manufacturer's instructions.

c) Analysis of surface expression: FACS. Approximately $5X10^5$ Jurkat cells were stained with 1μg/ml FITC labelled antigen or antibody specific for the binding component of the chimeric receptor. For analysis of receptors with a P67scFV binding component, FITC-labelled CD33 antigen was employed. Fluorescence was analysed by a FACScan cytometer (Becton Dickinson).

d) Analysis of function: IL-2 production. $2X10^5$ cells were incubated at 37°C in 8% CO_2 for 20 hours in 96 well plates with target cells at an effector:target ratio of 1:1. Cell supernatants were then harvested and assayed for human IL-2 (R & D Systems Quantikine kit).

Where P67scFv was employed as the example of an extracellular binding domain, the target cells used were:

HL60 cells – a human cell line naturally expressing the antigen, CD33.

N.EE6 – a mouse myeloma (NS0) transfected with a control expression vector. These cells are used as a negative control target cell line.

N.CD33 - a mouse myeloma (NS0) transfected with an expression vector facilitating the expression of antigen CD33 on the cell surface.

Example 5. Results

The specific production of IL-2 by Jurkat cells expressing chimeric receptors, in response to antigen challenge (either by HL-60 or N.CD33 cells as indicated), is used as a measure of signalling capability and the degree of cellular activation in all of the experiments described below.

a) Comparison of signalling capabilities of chimeric receptors with different primary signalling motifs (Figure 5).

N.CD33 cells were used to provide an antigen challenge to Jurkat cells expressing chimeric receptors with primary signalling motifs that vary in their inter-tyrosine distance. The results (shown in Figure 5) demonstrate that chimeric receptors with primary signalling motifs that have a longer (9 amino acid) spacer between YXXL motifs, as in SB16, are better at signalling than those with an average natural distance (7 amino acids) as in SB14.

b) Signalling through a chimeric receptor with an expanded primary signalling motif and an additional primary signalling motif. (Figure 6).

HL60 cells were used to provide an antigen challenge to Jurkat cells expressing a chimeric receptor with an expanded primary signalling motif. The results, illustrated in Figure 6, show the extremely efficient production of IL-2 by cells expressing such novel chimeric receptors. In this particular instance the primary signalling motif $SB3^a$ has been included upstream of the expanded motif SBX^a, to generate the highly efficient receptor P67scFV/h.CD28/CD28tm/SB3.SBX.

c) Signalling through a chimeric receptor with an expanded primary signalling motif and a secondary signalling sequence (Figure 7).

Jurkat cells expressing chimeric receptors comprising the novel primary signalling motifs, $SBQ5^a$, $SBQ7^a$ and $SBQ9^a$, as well as the secondary signalling sequence $SB28^a$, were challenged with N.CD33 cells. All chimeric receptors are capable of signalling however, a longer (9 amino acids), rather than shorter (5 amino acids), spacer between YXXL motifs in the primary signalling motifs again shown to be more efficacious than a spacer of average natural length (7 amino acids).

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CLAIMS

1. A nucleic acid encoding a cytoplasmic signalling sequence, which comprises a primary signalling motif that has the consensus amino acid sequence of:

$Y\text{-}X_2\text{-}L/I\text{-}X_n\text{-}Y\text{-}X_2\text{-}L/I$

wherein amino acid residues are represented by the standard single letter code, X represents any amino acid, a subscripted number indicates the number of residues present at that position within the motif and the value n is 9 or greater.

2. A nucleic acid according to claim 1, wherein the value of n lies between 9 and 12.

3. A nucleic acid according to claim 1, wherein the value of n is 9.

4. A nucleic acid according to claim 3, wherein the primary signalling motif has the amino acid sequence RKNPQEGLYNELQKDKMAEDTYDALHMQA (designated SBX^a), GQNQLYNELQQQQQQQQYDVLRRGRDPEM (designated $SBQ9^a$), or GQDGLYQELNTRSRDEAAYSVLEGR KAR (designated $SB16^a$).

5. A nucleic acid encoding a cytoplasmic signalling molecule comprising a primary signalling motif according to any one of the previous claims and at least one other primary signalling motif.

6. A nucleic acid according to claim 5, wherein the at least one primary other signalling motif comprises the consensus amino acid sequence: $Y\text{-}X_2\text{-}L/I\text{-}X_n\text{-}Y\text{-}X_2\text{-}L/I$.

7. A nucleic acid according to claim 6, wherein the value of n in the at least one other primary signalling motif is 9.

8. A nucleic acid according to claim 7, wherein the at least one other primary signalling motif is selected from: $SBQ9^a$, SBX^a, $SB16^a$.

9. A nucleic acid according to claim 6, wherein the value of n in the at least one other primary signalling motif lies between 6 and 8.

10. A nucleic acid according to claim 9, wherein the a least one other primary signalling motif comprises at least part of an immunoreceptor tyrosine based activation motif derived from the TCRζ chain, FcRγ, FcRβ, CD3γ, CD3δ, CD3ε, CD5, CD22, CD79a, CD79b or CD66d.

11. A nucleic acid according to claim 9, wherein at the least one additional primary signalling motif is $SB4^{*a}$, $SB14^{a}$, $SB15^{a}$.

12. A nucleic acid according to claim 10, wherein the cytoplasmic signalling molecule comprises SBX^{a} and $SB3^{a}$.

13. A nucleic acid according to claim 11, wherein the cytoplasmic signalling molecule comprises $SB16^{a}$, $SB4^{*a}$, $SB2^{a}$ and $SB4^{a}$.

14. A nucleic acid encoding a cytoplasmic signalling molecule comprising a primary signalling motif according to any one of claims 1 to 4 and at least one secondary signalling sequence.

15. A nucleic acid according to claim 14, wherein the at least one secondary signalling sequence is derived from CD28, CD134 or CD154.

16. A nucleic acid according to claim 15, wherein the at least one secondary signalling sequence is RLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFA, MIETYNQTSPRSAATGLPISMK, or RRDQRLPPDAHKPPGGGSFRTPIQEEQADAHS.

17. A nucleic acid according to claim 14, wherein the primary signalling motif is $SBQ9^{a}$, SBX^{a} or $SB16^{a}$ and the at least one secondary signalling sequence is RLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFA.

18. A nucleic acid according to claim 17, wherein the primary signalling motif is SBQ9[a] and the at least one secondary signalling sequence is RLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFA.

19. A nucleic acid encoding a chimeric receptor protein that comprises an extracellular ligand-binding domain, a transmembrane domain and a cytoplasmic signalling domain, wherein the cytoplasmic signalling domain is encoded by a nucleic acid according to any one of the preceding claims.

20. A nucleic acid according to claim 19, wherein the extracellular ligand-binding domain comprises an antibody binding domain, or a fragment thereof.

21. A nucleic acid according to claim 19, wherein the extracellular ligand-binding domain is a Fab' fragment or a scFv.

22. A nucleic acid according to claim 21 wherein the extracellular ligand-binding domain is a scFv.

23. A nucleic acid according to any one of claims 19 to 22, wherein the transmembrane domain is derived from the α, β or ζ chain of the T-cell receptor, CD28, CD3ε, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 or CD154.

24. A nucleic acid according to claim 23, wherein the transmembrane domain is derived from CD28.

25. A nucleic acid according to any one of claims 19 to 22, wherein the transmembrane domain is synthetic.

26. A vector comprising a nucleic acid according to any one of the preceding claims.

27. A cytoplasmic signalling protein encoded by a nucleic acid according to any one of claims 1 to 18.

28. A chimeric receptor protein encoded by a nucleic acid according to according to any one of claims 19 to 25.

29. A nucleic acid according to any one of claims 1 to 25, or a vector according to claim 26, for use in therapy.

30. A cytoplasmic signalling protein according to claim 27, or a chimeric receptor protein according to claim 28, for use in therapy.

31. A composition comprising a cytoplasmic signalling protein according to claim 27, or a chimeric receptor protein according to claim 28, or a nucleic acid according to any one of claims 1 to 25, in conjunction with a pharmaceutically acceptable excipient.

32. The use of, a nucleic acid, a cytoplasmic signalling protein, a chimeric receptor protein or, a composition according to any one of the preceding claims, in the manufacture of a medicament for the treatment or prevention of disease in humans or in animals.

33. A host cell containing a nucleic acid according to any one of claims 1 to 25, or a vector according to claim 26.

34. A host cell expressing a cytoplasmic signalling protein according to claim 27, or a chimeric receptor protein according to claim 28.

FIGURE 1

CLONING CASSETTE FOR CONSTRUCTION OF CHIMERIC RECEPTORS WITH NOVEL SIGNALLING COMPONENTS

FIGURE 2

```
Range: 1 to 200

  >SpeI
   |
   |        10          20          30          40          50          60
   |*       *      *     *      *      *     *      *      *     *      *      *     *
CG ACT AGT GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA AAA GGG AAA CAC CTT TGT CCA AGT CCC
GC TGA TCA CTG TTT TGA GTG TGT ACG GGT GGC ACG GGT TTT CCC TTT GTG GAA ACA GGT TCA GGG
    T   S   D   K   T   H   T   C   P   P   C   P   K   G   K   H   L   C   P   S  P>
                                                                              A6083

                              >NarI
  S0146                        |
   70          80          90|         100         110         120         130
    *      *     *      *     *|    *      *      *     *      *      *     *     *
CTA TTT CCC GGA CCT TCT AAG CCC GGC GCC TTT TGG GTG CTG GTG GTG GTT GGT GGA GTC CTG GCT
GAT AAA GGG CCT GGA AGA TTC GGG CCG CGG AAA ACC CAC GAC CAC CAC CAA CCA CCT CAG GAC CGA
 L   F   P   G   P   S   K   P   G   A   F   W   V   L   V   V   V   G   G   V   L  A>

                                                     >MluI  >BamHI
                           A6081                        |      |
            140         150         160         170     |  180 |     190
    *     *     *      *     *     *      *     *     * |   *  | *     *
TGC TAT AGC TTG CTA GTA ACA GTG GCC TTT ATT ATT TTC TGG GTG ACG CGT GGA TCC TGA
ACG ATA TCG AAC GAT CAT TGT CAC CGG AAA TAA TAA AAG ACC CAC TGC GCA CCT AGG ACT
 C   Y   S   L   L   V   T   V   A   F   I   I   F   W   V   T   R   G   S  *>
          A6082

>EcoRI
|
|       200
|  *     *
GAATTCATA
CTTAAGTAT
```

FIGURE 3

OLIGONUCLEOTIDE SEQUENCES FOR CHIMERIC RECEPTOR CONSTRUCTION
All oligos are listed in the 5' to 3' orientation

S0146	CGACTAGTGACAAAACTCACACATGCCCACCGTGCCCAAAAGGGAAACAC CTTTGTCCAACTCCC
A6081	GCCTTTTGGGTGCTGGTGGTGGTTGGTGGAGTCCTGGCTTGCTATAGCTT GCTAGTAACAGTG
A6082	TATGAATTCTCAGGATCCACGCGTCACCCAGAAAATAATAAAGGCCACTGT TACTAGCAAGCTATAG
A6083	CACCACCAGCACCCAAAAGGCGCCGGGCTTAGAAGGTCCGGGAAATAGG GGACTTGGAC
A8810	GATCCTGGTTTCTCATGCTTCAGAGTCTCGTAAGTCTCCTGGTTCCTGGTG CTCAGGCCCGTGTAACACCATCTGATTTCTCATAT
A8810B	GATCCTGGTTTCTCATGCTTCAGAGTATCGTAAGTCTCCTGGTTCCTGGTG CTCAGGCCCGTGTAACACCATCTGATTTCTCATAT
A8811	GATCATATGAGAAATCAGATGGTGTTTACACGGGCCTGAGCACCAGGAAC CAGGAGACTTACGAGACTCTGAAGCATGAGAAACCAG
A8811B	GATCATATGAGAAATCAGATGGTGTTTACACGGGCCTGAGCACCAGGAAC CAGGAGACTTACGATACTCTGAAGCATGAGAAACCAG
A8812	GATCCGGCCTGCATGTGAAGGGCGTCGTAGGTGTCCTTGGTGGCTGTACT GAGACCCTGGTAAAGGCCATCGTGCCCCTGTCCCCTT
A8813	GATCAAGGGGCAAGGGGCACGATGGCCTTTACCAGGGTCTCAGTACAGCC ACCAAGGACACCTACGACGCCCTTCACATGCAGGCCG
A8814	GATCCGCGCTCGCCTTTCATCCCAATCTCACTGTAGGCCTCCGCCATCTTA TCTTTCTGCAGTTCATTGTACAGGCCTTCCTGAGGGTTCTTCCTT
A8815	GATCAAGGAAGAACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGAT AAGATGGCGGAGGCCTACAGTGAGATTGGGATGAAAGGCGAGCGCG
A8816	GATCCCATCTCAGGGTCCCGGCCACGTCTCTTGTCCAAAACATCGTACTCC TCTCTTCGTCCTAGATTGAGCTCGTTATAGAGCTGGTTCTGGCCT
A8817	GATCAGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAG GAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGG
A9000	GATCAGGAAACAAGGTTCCAGAGGATCGTGTTTATGAAGAATTAAACATAT ATTCAGCTACTTACAGTGAGTTGGAAGACCCAGGGGAAATGTCTCCTG
A9001	GATCCAGGAGACATTTCCCCTGGGTCTTCCAACTCACTGTAAGTAGCTGAA TATATGTTTAATTCTTCATAAACACGATCCTCTGGAACCTTGTTTCCT
A9002	GATCAAAGCAGACTCTGTTGCCCAATGACCAGCTCTACCAGCCCCTCAAG GATCGAGAAGATGACCAGTACAGCCACCTTCAAGGAAACCAGTTGAGGG
A9003	GATCCCCTCAACTGGTTTCCTTGAAGGTGGCTGTACTGGTCATCTTCTCGA TCCTTGAGGGGCTGGTAGAGCTGGTCATTGGGCAACAGAGTCTGCTTT
A9004	GATCAGCTCTGTTGAGGAATGACCAGGTCTATCAGCCCCTCCGAGATCGA GATGATGCTCAGTACAGCCACCTTGGAGGAAACTGGGCTCGGAACAAGG
A9005	GATCCCTTGTTCCGAGCCCAGTTTCCTCCAAGGTGGCTGTACTGAGCATCA TCTCGATCTCGGAGGGGCTGATAGACCTGGTCATTCCTCAACAGAGCT
A9006	GATCACAAAACAAGGAGAGGCCACCACCTGTTCCCAACCCAGACTATGAG CCCATCCGGAAAGGCCAGCGGGACCTGTATTCTGGCCTGAATCAGAGACG CATCG

FIGURE 3 continued

A9007 GATCCGATGCGTCTCTGATTCAGGCCAGAATACAGGTCCCGCTGGCCTTT
CCGGATGGGCTCATAGTCTGGGTTGGGAACAGGTGGTGGCCTCTCCTTGT
TTTGT

A9008 GATCACACGTGGATAACGAATACAGCCAACCTCCCAGGAACTCCCGCCTG
TCAGCTTATCCAGCTCTGGAAGGGGTTCTGCATCGCTCCG

A9009 GATCCGGAGCGATGCAGAACCCCTTCCAGAGCTGGATAAGCTGACAGGCG
GGAGTTCCTGGGAGGTTGGCTGTATTCGTTATCCACGTGT

A9010 GATCACCTCCCCGGACCTGCGATGACACGGTCACTTATTCAGCATTGCACA
AGCGCCAAGTGGGCGACTATGAGAACGTCATTCCAGATTTTCCAGAAGAT
GAGG

A9011 GATCCCTCATCTTCTGGAAAATCTGGAATGACGTTCTCATAGTCGCCCACT
TGGCGCTTGTGCAATGCTGAATAAGTGACCGTGTCATCGCAGGTCCGGGG
AGGT

A9012 GATCAGAATATGAAGATGAAAACCTTTATGAAGGCCTGAACCTGGACGACT
GCTCCATGTATGAGGACATCTCCCGGGGCCTCCAGGGCACCTACCAGGAT
GTGG

A9013 GATCCCACATCCTGGTAGGTGCCCTGGAGGCCCCGGGAGATGTCCTCATA
CATGGAGCAGTCGTCCAGGTTCAGGCCTTCATAAAGGTTTTCATCTTCATA
TTCT

A9014 GATCAAAGGCTGGCATGGAGGAAGATCACACCTACGAGGGCCTGGACATT
GACCAGACAGCCACCTATGAGGACATAGTGACGCTGCGGACAGGGGAAG
TGG

A9015 GATCCCACTTCCCCTGTCCGCAGCGTCACTATGTCCTCATAGGTGGCTGTC
TGGTCAATGTCCAGGCCCTCGTAGGTGTGATCTTCCTCCATGCCAGCCTTT

A9016 GATCACCCCTACCCAACCCCAGGACAGCAGCTTCCATCTATGAGGAATTGC
TAAAACATGACACAAACATTTACTGCCGGATGGACCACAAAGCAGAAGTGG
CTG

A9017 GATCCAGCCACTTCTGCTTTGTGGTCCATCCGGCAGTAAATGTTTGTGTCA
TGTTTTAGCAATTCCTCATAGATGGAAGCTGCTGTCCTGGGGTTGGGTAGG
GGT

A9018 GATCAAGGCTCCTGCACAGTGACTACATGAACATGACTCCTCGCCGACCA
GGGCCAACCCGCAAGCATTACCAGCCCTATGCCCCACCACGCGACTTCGC
AG

A9019 GATCCTGCGAAGTCGCGTGGTGGGGCATAGGGCTGGTAATGCTTGCGGG
TTGGCCCTGGTCGGCGAGGAGTCATGTTCATGTAGTCACTGTGCAGGAGC
CTT

A9020 GATCAATGATCGAAACATACAACCAAACTTCTCCCCGATCTGCGGCCACTG
GACTGCCCATCAGCATGAAAG

A9021 GATCCTTTCATGCTGATGGGCAGTCCAGTGGCCGCAGATCGGGGAGAAGT
TTGGTTGTATGTTTCGATCATT

F6392 GATCAGGACAGGACGGTCTATACCAGGAGCTGAACACCAGGTCGCGGGAT
GAGGCCTACTCGGTCCTAGAAGGCCGCAAGGCAAGAG

F6393 GATCAGGACAGGACGGTCTATACCAGGAGCTGAACACCAGGTCGCGGGAT
GAGGCCGCCTACTCGGTCCTAGAAGGCCGCAAGGCAAGAG

D5258 GATCAGGACAGGACGGTCTATACCAGGAGCTGAACACCAGGTCGCGGGAT
GAGTACTCGGTCCTAGAAGGCCGCAAGGCAAGAG

D5259 GATCCTCTTGCCTTGCGGCCTTCTAGGACCGAGTACTCATCCCGCGACCT
GGTGTTCAGCTCCTGGTATAGACCGTCCTGTCCT

F6394 GATCCTCTTGCCTTGCGGCCTTCTAGGACCGAGTAGGCCTCATCCCGCGA
CCTGGTGTTCAGCTCCTGGTATAGACCGTCCTGTCCT

FIGURE 3 continued

F6395 GATCCTCTTGCCTTGCGGCCTTCTAGGACCGAGTAGGCGGCCTCATCCCG
CGACCTGGTGTTCAGCTCCTGGTATAGACCGTCCTGTCCT

D7609 GATCACAGAACCAGCTCTATAACGAGCTCCAGCAGCAGCAGCAGTACGAT
GTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGG

D7610 GATCCCATCTCAGGGTCCCGGCCACGTCTCTTGTCCAAAACATCGTACTGC
TGCTGCTGCTGGAGCTCGTTATAGAGCTGGTTCTGT

D7613 GATCACAGAACCAGCTCTATAACGAGCTCCAGCAGCAGCAGCAGCAACAG
TACGATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGG

D7614 GATCCCATCTCAGGGTCCCGGCCACGTCTCTTGTCCAAAACATCGTACTGT
TGCTGCTGCTGCTGGAGCTCGTTATAGAGCTGGTTCTGT

D7617 GATCACAGAACCAGCTCTATAACGAGCTCCAGCAGCAGCAGCAGCAACAG
CAACAATACGATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGG

D7618 GATCCCATCTCAGGGTCCCGGCCACGTCTCTTGTCCAAAACATCGTATTGT
TGCTGTTGCTGCTGCTGCTGCTGGAGCTCGTTATAGAGCTGGTTCTGT

F1340A GATCACGGAGGGACCAGAGGCTGCCCCCCGATGCCCACAAGCCCCCTGG
GGGAGGCAGTTTCCGGACCCCCATCCAAGAGGAGCAGGCGGACGCCCAC
TCCACCCTGGCCAAGATCG

F1340B GATCCGATCTTGGCCAGGGTGGAGTGGGCGTCGGCCTGCTCCTCTTGGAT
GGGGGTCCGGAAACTGCCTCCCCCAGGGGGCTTTTGGGCATCGGGGGGC
AGCCTCTGGTCCCTCCGT

FIGURE 4

THE AMINO ACID SEQENCE OF SEQUENCE BLOCKS

The single letter amino acid code is used

SBQ5: GSGQNQL**YNEL**QQQQQ**YDVL**RRGRDPEMGS
SBQ7: GSGQNQL**YNEL**QQQQQQQ**YDVL**RRGRDPEMGS
SBQ9: GSGQNQL**YNEL**QQQQQQQQQQ**YDVL**RRGRDPEMGS

SBX: GSRKNPQEGL**YNEL**QKDKMAEDT**YDAL**HMQAGS

SB1: GSGQNQL**YNEL**NLGRREE**YDVL**DKRRGRDPEMGS
SB2: GSRKNPQEGL**YNEL**QKDKMAEA**YSEI**GMKGERGS
SB3: GSRGKGHDGL**YQGL**STATKDT**YDAL**HMQAGS
SB4: GSYEKSDGV**YTGL**STRNQET**YETL**KHEKPGS
SB4*: GSYEKSDGV**YTGL**STRNQET**YDTL**KHEKPGS
SB5: GSGNKBPEDRV**YEEL**NIYSAT**YSEL**EDPGEMSPGS
SB6: GSKQTLLPNDQL**YQPL**KDREDDQ**YSHL**QGNQLRGS
SB7: GSALLRNDQV**YQPL**RDRDDAQ**YSHL**GGNWARNKGS
SB8: GSQNKERPPPVPNPD**YEPI**RKGQRDL**YSGL**NQRRIGS
SB9: GSHVDNE**YSQP**PRNSRLSA**YPAL**EGVLHRSGS
SB10: GSPPRTCDDTVT**YSAL**HKRQVGD**YENV**IPDFPEDEGS
SB11: GSEYEDENL**YEGL**NLDDCSM**YEDI**SRGLQGTYQDVGS
SB12: GSKAGMEEDHT**YEGL**DIDQTAT**YEDI**VTLRTGEVGS
SB13: GSPLPNPRTAASI**YEEL**LKHDTNI**YCRM**DHKAEVAGS

SB14: GSGQDGL**YQEL**NTRSRDE**YSVL**EGRKARGS
SB15: GSGQDGL**YQEL**NTRSRDEA**YSVL**EGRKARGS
SB16: GSGQDGL**YQEL**NTRSRDEAA**YSVL**EGRKARGS

SB28: GSRLLHSD**YMNM**TPRRPGPTRKHYQPYAPPRDFAGS
SB29: GSMIET**YNQT**SPRSAATGLPISMKGS
SB34: GSRRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKIGS

FIGURE 5

ANTIGEN SPECIFIC STIMULATION OF CHIMERIC RECEPTORS WITH PRIMARY SIGNALLING MOTIFS OF VARYING INTER-TYROSINE DISTANCE

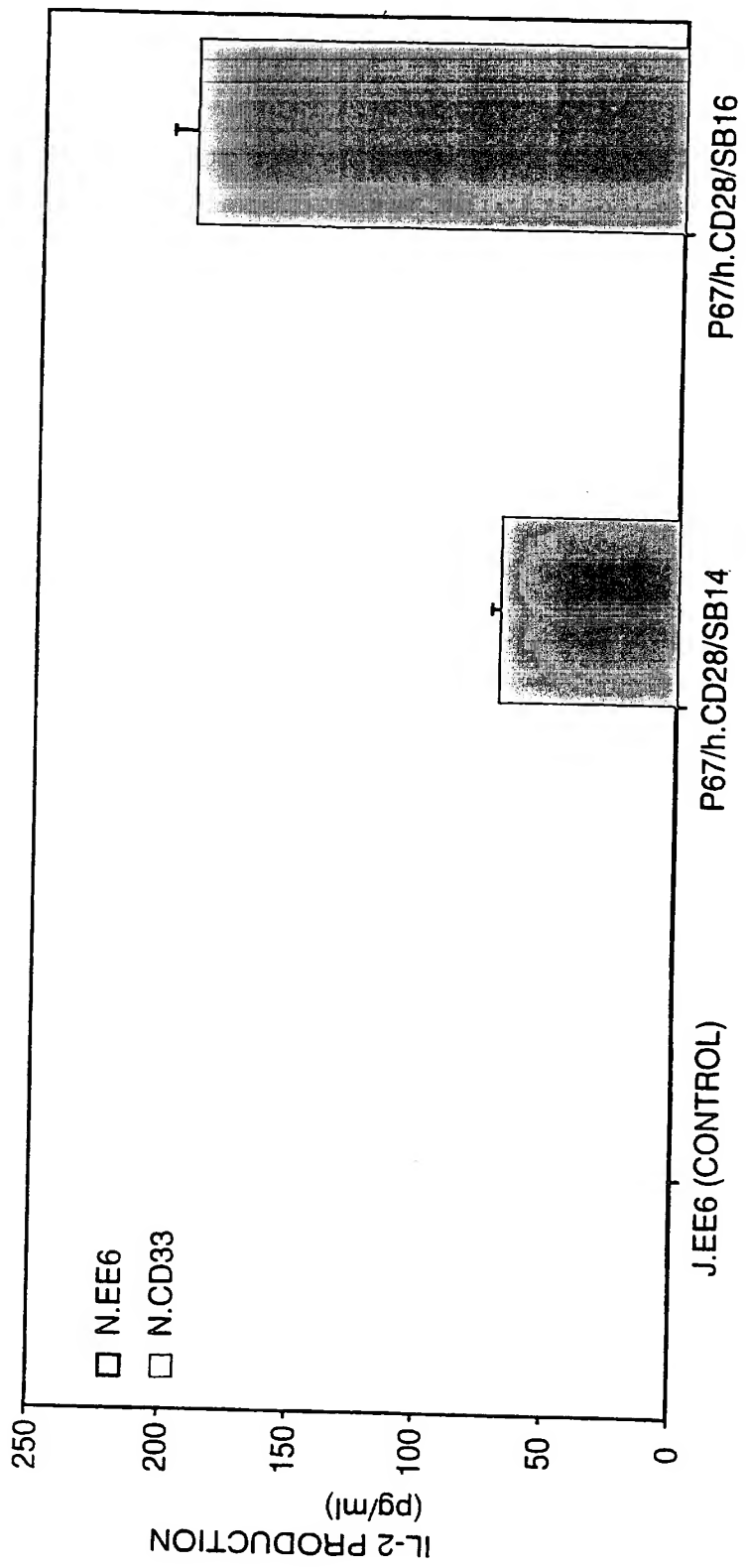


FIGURE 6

ANTIGEN SPECIFIC STIMULATION OF A CHIMERIC RECEPTOR WITH AN EXPANDED PRIMARY SGNALLING MOTIF IN CONJUNCTION WITH A PRIMARY SIGNALLING MOTIF

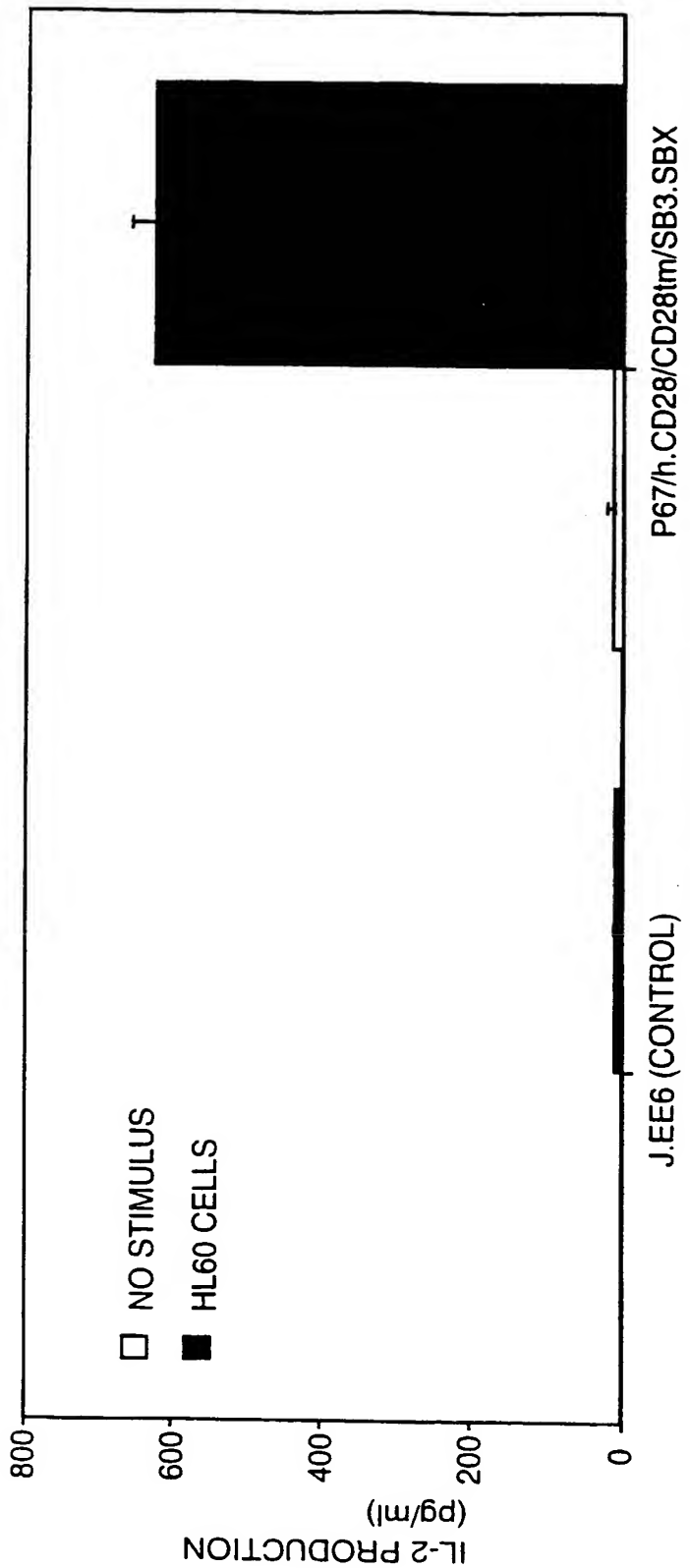
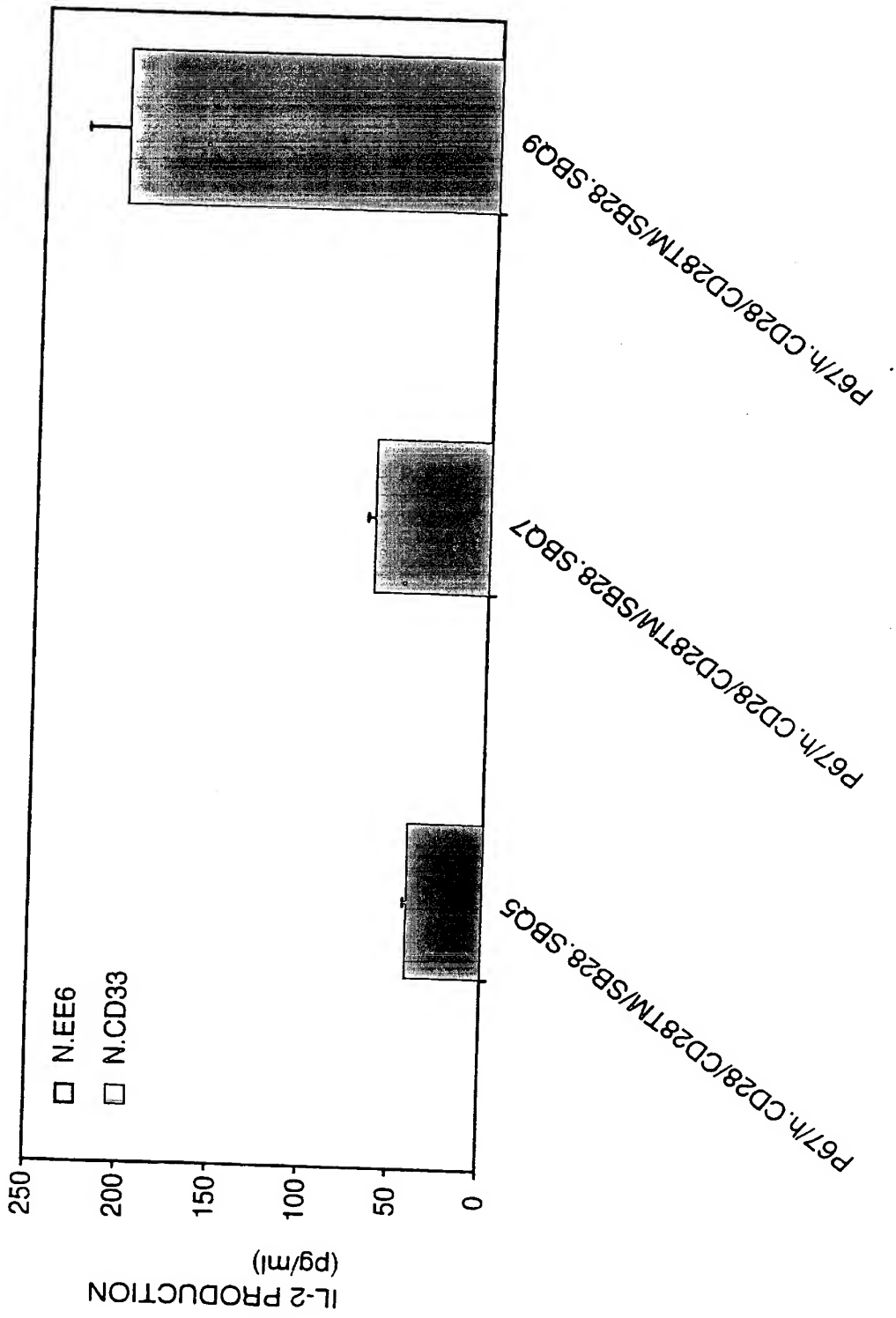


FIGURE 7

ANTIGEN SPECIFIC STIMULATION OF CHIMERIC RECEPTORS COMPRISING PRIMARY SIGNALLING MOTIFS OF VARYING INTER-TYROSINE DISTANCE WITH A SECONDARY SIGNALLING SEQUENCE



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/04193

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C07K14/705 C12N15/62 C12N5/10 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, STRAND, EMBL, CHEM ABS Data, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 23613 A (BEBBINGTON CHRISTOPHER ROBERT ;CELLTECH THERAPEUTICS LTD (GB); FIN) 3 July 1997 (1997-07-03) cited in the application claims 1-52; figures 1,2,4,14-17; examples 1-8	1,5,6,9, 10, 12-15, 19-24, 26-34
X	US 5 851 828 A (ROMEO CHARLES ET AL) 22 December 1998 (1998-12-22) SEQ ID NO: 37 abstract; claims 1-15; figures 7,9,11,12,20; examples 1-12	1,5,6,9, 10,12, 13,19, 23,26-34

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
9 March 2001	21/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Devijver, K

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Int. ional Application No
PCT/GB 00/04193

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